Cortical Dynein Drives Centrosome Clustering in Cells with Centrosome Amplification

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RE: Manuscript #E22-07-0296
TITLE: Cortical Dynein Drives Centrosome Clustering in Cells with Centrosome Amplification

Dear Authors, one of the reviewers is very enthusiastic about the study; another is more critical. Please revise according to comments of both reviewers, and I will send the revised manuscript to the second reviewer for the second look.

Sincerely,

Alexander Mogilner
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Manning,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
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Reviewer #1 (Remarks to the Author):

This interesting paper highlights a role for cortically-localized dynein in facilitating the clustering of spindle poles in cells with multiple centrioles. Multi-polar spindles generated due to the presence of increased centriole numbers are a major challenge for chromosome instability, including during cancer progression. Division of a truly multi-polar spindle can be catastrophic for a cell, but in most cases a cell is able to cluster these spindle poles into two foci to create a bipolar spindle (albeit with chromosome

mis-segregation issues that result). Prior work has focused on the spindle proteins that drive this clustering behavior, including the kinesin motor HSET/KifC1 and others. Instead of looking internal to the spindle, this paper presents an additional model for how this clustering is achieved through the forces created by dynein at the cell cortex. To my knowledge, this is the first time that the cortical dynein has been implicated in this process (instead of in spindle positioning and orientation, or spindle elongation). By precisely eliminating cortical dynein (including using LGN depletion and other perturbations), the authors show that this is the case. The data in Figures 2 and 3 is incredibly clear and compelling. In addition to using fixed and live cell imaging to analyze this question, the authors also conduct mathematical modeling to evaluate the potential contributions of cortical force and restricted dynein localization in driving spindle pole clustering.

I really liked this paper, and I believe that it is a great fit for MBOC. The idea is new and compelling, and the results are very clear from the presented data. I think that this is an important advance for the mechanisms of spindle formation and the roles of cortical dynein. I would support publication of this paper largely as is. Thus, my comments are more conceptual and stylistic, and I defer to the authors for these changes. Overall, I congratulate the authors on the excellent and exciting work.

• I found the findings from the computational modeling approaches to be very interesting, although I have a harder time evaluating the approaches based on my background. However, at present, it does sometimes feel that this is two different papers (an experimental paper followed by a computational paper). Considering ways to integrate these aspects of the paper could be useful.
• The experimental data is very clear to highlight a role for cortical dynein in spindle pole clustering. The computational modeling attempts to take this one step further to suggest an important role for localized zones of dynein (in a region behind the spindle pole) and the oscillatory re-localization of dynein. These are features of dynein localization that have been observed in prior work, but they are very difficult things to test experimentally (for example, would need ontogenetic targeting of dynein to the cortex as has been done by Kiyomitsu and co-workers - something that is beyond the scope of this paper). Thus, I would use some caution with these points, and considering the way that this is framed in the text to highlight the need to test these points in the future may be useful. Similarly, the wording of the figure legend titles for these two figures seems more definitive than I think this work can claim (maybe "Computational modeling suggests ..." instead).
• Although LGN plays a role in cortical dynein recruitment, it is actually not essential in cell culture in most cell lines (based on DepMap). Here, the finding that it is required for centrosome clustering appears very potent suggesting that disrupting this pathway in selected cancer cells could be useful. The frequency of multipolar spindles in the LGN depleted cells appears high such that this should compromise viability. It would be a very powerful result if the authors could show that cells displayed differential viability/growth requirements for LGN depending on the number of centrioles (a synergy). This would possibly need to be done in some type of sensitized growth assay, and I respect that this may be beyond the scope of this paper, but showing genetically that this is true for predicting cell proliferation behaviors would be a highly impactful finding.

Reviewer #2 (Remarks to the Author):

In this study, the authors use a combination of experimental and modeling approaches to investigate the role of cortical dynein in promoting centrosome clustering in cells with supernumerary centrosomes. Understanding the mechanisms of centrosome clustering is important because this may help identify potential therapeutic targets for cancers with centrosome amplification. Unfortunately, there are some issues with the experimental data that raise substantial concerns. And because some of the modeling work is based on the authors' experimental observations, the concerns on the experimental data also affect the relevance of the modeling work. Below is a list of both major and minor issues.

Major issues:

1. The authors use NuMA staining to infer localization of cortical dynein. Unfortunately, the quality of the images presented here is simply not sufficient for this analysis to be reliable. Moreover, the localization observed here is inconsistent with what was reported in other studies, including some cited here (e.g., Kiyomitsu and Cheeseman, 2012 and Seldin et al., 2013). It is possible that these differences between studies may be due to differences between cell types. However, because the quality of the images here is so poor, it is impossible to tell. And if better quality images cannot be obtained in the cell types used here, then a better experimental model should have been used. Or perhaps the immunostaining could have been better optimized. Regardless of the image quality, the authors should perform a more quantitative type of analysis to better determine the region(s) of the cortex occupied by NuMA (hence, dynein). Importantly, in other studies, NuMA was shown to concentrate on one side of the cell, but to occupy about half of the cell cortex, instead of a quadrant as suggested here. Because an assumption of the model is that cortical dynein occupies a quadrant, the concerns about the experimental data also extend to the model.

2. Another major concern is that in a lot of images (and in the model), the authors present cells with three centrosomes. However, in all their DCB-treated cells, four centrosomes would be expected. Moreover, in some cases there appear to be centrosomes with a single centrin dot. It is not clear if that is again, just poor quality of the images or a real phenotype, in which case an explanation should be provided or the experimental design revisited, because in all DCB-treated cells one would expect four centrosomes with two centrin dots each. And in the case of Plk4 overexpression one would expect even more centrosomes, but information about how much centrosome amplification was achieved is not discussed. Moreover, neither image shown in Figure 2C is a really good example of an MDA-MB-231 cell w/ extra centrosomes. The top image appears to have a pole with an

extra centriole and in the bottom image, it is hard to see whether the spindle poles on the left or bottom of the image have two centrioles - instead it looks like it could be an issue of centrosome splitting in a cell with the correct number of centrioles. Overall, the experimental data on centrosome number is not sufficiently detailed or of high-enough quality to ensure that the modeling work was performed with biologically relevant assumptions/parameters. Finally, the authors do not provide details on how centrosome clustering is defined. For instance, in simulations with four centrosomes, is clustering always 2-2 or can it be 3-1? This should be defined both in the experiments and in the simulations.

3. At one point in the manuscript, the authors bring up the observation that dynein can oscillate between the two poles of the cell. It would be useful for this information to be provided earlier. In the simulations, this oscillation is generated by moving the dynein parameters to opposite ends of the simulated circle every few minutes (varied by simulation). What if dynein oscillated between three or four positions? Would this lead to multipolar cells? And does dynein only oscillate after the poles are formed? If so, then is cortical dynein really important for centrosome clustering or is it important just for maintaining bipolarity? Also, the simulations with oscillating dynein are only performed with three centrosomes. It would be important to do this with 4+ centrosomes.

4. One data set in the manuscript was obtained by imaging live cells expressing fluorescently-tagged tubulin. This, again, seems to be not ideal because information about centrosome number, which is important in the context of this study, is not available in these experiments. The authors argue that these live-cell experiments are necessary to understand whether centrosome clustering occurs late in metaphase (but prior to anaphase onset), which may be underestimated by the fixed-cell analysis. However, analyzing centrosome clustering in both late prometaphase/metaphase and anaphase cells in fixed samples immunostained for tubulin and centrin would provide more informative data than the live-cell experiments presented here.

5. Can the authors show IF images that confirm/show how dynein localization is disrupted after Afadin and LGN KD? Is dynein/NuMa distributed around the entire cortex or entirely absent? Are dynein oscillations affected? All this would be useful information for the model.

6. Some of the results in Fig. 3 are difficult to reconcile. Specifically, after 1 hr in MG132 only ~80% of RPE1 indPLK4 have bipolar (clustered) spindles, but in the live cell images experiments the average mitotic duration is 45 min (shorter than the 1 hr MG132 treatement) and ~95% of the cells achieve bipolarity. Also, these numbers seem high for RPE1 cells and inconsistent with findings from other studies (e.g., Chen at al., Mol Biol Cell, 2016 and Baudoin et al., eLife, 2020). Is the level of clustering being overestimated here or is this specific to PLK4-induced centrosome amplification compared to DCB or endoreduplication?

7. The model predicts that dynein/NuMA oscillations could be important for clustering centrosomes. GFP-tagged NuMA and live cell imaging could help to test this. Even the current live-cell experiments could be used. The model would suggest that centrosomes closer to the Dynein/NuMA pole would cluster first; then, upon oscillation the other centrosomes may start to cluster. Is there any evidence for this (i.e., spatial/temporal differences in clustering of centrosomes) in the time-lapse videos?

Minor issue

1. There were many issues with figure/panel numbering and descriptions in figure legends. For instance, some cited figure panels did not exist, figures were note referenced in order, some panels were not described in the figure legends, etc. There were also some labeling issues in the figure themselves (e.g., MDA-MB-231 labeled as MDA-MB-123 in Fig. 2F).

2. Line 184, the text "with significant decreases in the pairwise distance between centrosomes to which those microtubules are anchored for the case of dynein in an angular region of pi/2" is not clear.

3. Line 281-283. The statement "the formation of a bipolar spindle with a given region of dynein enrichment is not sensitive to centrosome number" is not sufficiently supported by the data provided. Simulation data should be provided to show what happens when the number of centrosomes is higher than four. It would also be important to show whether the "optimal" dynein enriched region changes when centrosome number increases (like in figure S4E)

4. Model parameters: how were the values for P_E and P_H chosen? Specifically, why is P_H set to 0.7 and not to 0.5 like P_E? Is there any experimental evidence for such a difference between Eg5 and HSET?

5. Figure 4A. It would be helpful to label/mark the 0~pi/2 dynein enriched region.

We thank both reviewers for their enthusiastic and thoughtful critique. We have fully addressed each comment, as detailed below, and feel that the experimental and textual changes suggested by reviewers have improved the clarity of the manuscript. Additions to the discussion now better represent our findings- including both strengths and limitations of our approaches, in the broader context of the field.

Point by point response to reviewers:

Reviewer #1 (Remarks to the Author):
This interesting paper highlights a role for cortically-localized dynein in facilitating the clustering of spindle poles in cells with multiple centrioles. Multi-polar spindles generated due to the presence of increased centriole numbers are a major challenge for chromosome instability, including during cancer progression. Division of a truly multi-polar spindle can be catastrophic for a cell, but in most cases a cell is able to cluster these spindle poles into two foci to create a bipolar spindle (albeit with chromosome mis-segregation issues that result). Prior work has focused on the spindle proteins that drive this clustering behavior, including the kinesin motor HSET/KifC1 and others. Instead of looking internal to the spindle, this paper presents an additional model for how this clustering is achieved through the forces created by dynein at the cell cortex. To my knowledge, this is the first time that the cortical dynein has been implicated in this process (instead of in spindle positioning and orientation, or spindle elongation). By precisely eliminating cortical dynein (including using LGN depletion and other perturbations), the authors show that this is the case. The data in Figures 2 and 3 is incredibly clear and compelling. In addition to using fixed and live cell imaging to analyze this question, the authors also conduct mathematical modeling to evaluate the potential contributions of cortical force and restricted dynein localization in driving spindle pole clustering.

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We appreciate the reviewer's concern. However, because the computational model is informed by the results of our experimental work, we found it difficult to reorganize the presentation of results. Instead, to address this reviewer's concern and better integrate the computational and experimental approaches, we now highlight within the text where computational and experimental data are in concordance and where computational approaches expand upon what is feasible (within the scope of this study) in an experimental system. These changes are reflected throughout both the results conclusions sections.

• The experimental data is very clear to highlight a role for cortical dynein in spindle pole clustering. The computational modeling attempts to take this one step further to suggest an important role for localized zones of dynein (in a region behind the spindle pole) and the oscillatory re-localization of dynein. These are features of dynein localization that have been observed in prior work, but they are very difficult

things to test experimentally (for example, would need ontogenetic targeting of dynein to the cortex as has been done by Kiyomitsu and co-workers - something that is beyond the scope of this paper). Thus, I would use some caution with these points, and considering the way that this is framed in the text to highlight the need to test these points in the future may be useful. Similarly, the wording of the figure legend titles for these two figures seems more definitive than I think this work can claim (maybe "Computational modeling suggests ..." instead).

We agree with this reviewer that it would be very exciting for future work to test some of the interpretations of our data using optogenetic approaches to target dynein to defined regions of the cortex and have expanded our discussion accordingly. We have also revised the text and figure legends to better reflect and not overstate the conclusions directly supported by our data.

• Although LGN plays a role in cortical dynein recruitment, it is actually not essential in cell culture in most cell lines (based on DepMap). Here, the finding that it is required for centrosome clustering appears very potent suggesting that disrupting this pathway in selected cancer cells could be useful. The frequency of multipolar spindles in the LGN depleted cells appears high such that this should compromise viability. It would be a very powerful result if the authors could show that cells displayed differential viability/growth requirements for LGN depending on the number of centrioles (a synergy). This would possibly need to be done in some type of sensitized growth assay, and I respect that this may be beyond the scope of this paper, but showing genetically that this is true for predicting cell proliferation behaviors would be a highly impactful finding.

We agree that further characterization of the potential synthetic interactions between cortical dynein inhibition (via LGN depletion) and centrosome number would be a very exciting extension of our work and we hope to explore this in both cancer cell lines and animal models. However, as this reviewer suggested, this direction is beyond the scope of the current study.

Reviewer #2 (Remarks to the Author):

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NuMA (hence, dynein). Importantly, in other studies, NuMA was shown to concentrate on one side of the cell, but to occupy about half of the cell cortex, instead of a quadrant as suggested here. Because an assumption of the model is that cortical dynein occupies a quadrant, the concerns about the experimental data also extend to the model.

While we can not rule out the possibility that cell type or organism of origin may impact the robustness of NuMA localization to the cortex (the Seldin et al paper used mouse keratinocytes), we respectfully disagree with the reviewer that our capacity to monitor endogenous NuMA localization at the cell cortex of human cells is lacking, compared to previous works in cultured cell lines. Indeed, as this reviewer suggest, images in the manuscript by Kiyomitsu and Cheeseman depicting cortex localization of the LGN/NuMA/dynein complex are beautifully represented. However, the Kiyomitsu and Cheeseman study primarily used mCherry or GFP-tagged overexpression constructs to enable the robust cortical staining patterns shown. In the images assessing endogenous NuMA at the cortex, we find the staining similar to ours (please see Kiyomitsue and Cheeseman supplemental figures 2a and 5b). Nevertheless, to better represent the staining of NuMA on the cell cortex we now utilize NIS Elements clarify module to remove blurred light, resulting in more high contrast images. We have also expanded our discussion to highlight apparent differences between cell types and comment on the potential implications of differences in NuMA distribution on the cortex.

Consistent with differences in NuMA intensity along the cortex, distribution of NuMA also appears to differ between keratinocytes/overexpression in human cells (Seldin et al, Kiyomitsu and Cheeseman, repectively) and endogenous NuMA, with the former occupying a larger region of the cortex. Similar to this reviewer, we too were curious how these differences in distribution of NuMA along the cortex may (or may not) impact the clustering capacity of a cell with extra centrosomes and we performed modeling to assess a range of NuMA/dynein distributions. This data was represented in Supplemental Figure 4 where we used computational models to examine centrosome clustering when NuMA localization is restricted to a cortical region the size of pi/8, pi/4, pi/2, or 3pi/4, or instead is uniformly distributed over the cell cortex. These data indicate that there is a range of NuMA distribution (pi/4 to 3pi/4) that is consistent with the range of NuMA distribution presented in the literature that support centrosome clustering. We have revised the text to better highlight these data.

2. Another major concern is that in a lot of images (and in the model), the authors present cells with three centrosomes. However, in all their DCB-treated cells, four centrosomes would be expected. Moreover, in some cases there appear to be centrosomes with a single centrin dot. It is not clear if that is again, just poor quality of the images or a real phenotype, in which case an explanation should be provided or the experimental design revisited, because in all DCB-treated cells one would expect four centrosomes with two centrin dots each.

We have added insets to each panel representing a DCB-treated cell to clarify that these cells do indeed each have four pairs of centrioles.

And in the case of Plk4 overexpression one would expect even more centrosomes, but information about how much centrosome amplification was achieved is not discussed.

The methods section and illustrated timeline in Supplemental Figure 1 describes both the timeline and concentration of doxycycline used to induce PLK4 expression in order to promote centriole duplication. This is a well-established system (Godhino et al 2009; Ganem et al., 2009) that promotes robust centriole overduplication by the second mitosis following PLK4 overexpression. RPE cells have a cell

cycle timing of ~17-22h, making our 48h induction timeline sufficient to ensure all mitotic cells have undergone a previous mitosis in the presence of overexpressed PLK4.

Moreover, neither image shown in Figure 2C is a really good example of an MDA-MB-231 cell w/ extra centrosomes. The top image appears to have a pole with an extra centriole and in the bottom image, it is hard to see whether the spindle poles on the left or bottom of the image have two centrioles - instead it looks like it could be an issue of centrosome splitting in a cell with the correct number of centrioles.

We primarily observe centrosome amplification in these cells and these data are represented in Supplemental Figure 1E (formerly Supplemental Figure F). Insets have been added to the figures to better illustrate the presence of duplicated centriole pairs. However, given the MDA-MB-231 cells exhibit a number of mitotic defects, it is possible that individual cells within the population may exhibit either one or both centrosome amplification and centriole splitting. We have revised the text to better reflect this possibility. Regardless, we believe these cells are an appropriate system in which to assess the role of LGN and Afadin in spindle bipolarity as they are able to efficiently cluster their extra centrosomes/centrioles during mitotic progression.

Overall, the experimental data on centrosome number is not sufficiently detailed or of high-enough quality to ensure that the modeling work was performed with biologically relevant assumptions/parameters.

Our model considers centrosomes as a microtubule nucleating and organizing center and does not distinguish between a centrosome pair and individual centrioles. As such, the ability of our model to reflect focusing activity is dependent on number of microtubule organizing centers and irrespective of centriole number.

Finally, the authors do not provide details on how centrosome clustering is defined. For instance, in simulations with four centrosomes, is clustering always 2-2 or can it be 3-1? This should be defined both in the experiments and in the simulations.

We have defined clustering in both the results and methods sections. In the results section, we describe clustering as when “one or both spindle poles contained two or more centrosomes located within 5 um of each other”. To emphasize that these same parameters are reflected in the computational modeling approaches, this definition is restated several times throughout the text and reiterated in the methods section. We now also comment on the predominance of 3-1 clustering for the case of 4 centrosomes in the Discussion and relate this to other previously published results (Baudoin et al 2020).

3. At one point in the manuscript, the authors bring up the observation that dynein can oscillate between the two poles of the cell. It would be useful for this information to be provided earlier. In the simulations, this oscillation is generated by moving the dynein parameters to opposite ends of the simulated circle every few minutes (varied by simulation). What if dynein oscillated between three or four positions? Would this lead to multipolar cells? And does dynein only oscillate after the poles are formed? If so, then is cortical dynein really important for centrosome clustering or is it important just for maintaining bipolarity? Also, the simulations with oscillating dynein are only performed with three centrosomes. It would be important to do this with 4+ centrosomes.

In vivo data has not indicated that dynein oscillates between more than two positions. The model based on work by Kiyomitsu and Cheeseman, 2012 poses that cortical dynein both drives centrosome

positioning and is responsive to centrosome proximity (dynein pulls the spindle pole towards the cortex until NuMA/dynein is within the inhibitory PLK1-gradient generated at said centrosome). We now include data from computational modeling of dynein oscilations in simulations with 4 centrosomes. These simulation show a similar increase in clustering when the region of dynein enrichment oscillates, compared to stationary dynein enrichment (Figures 4 and 5). While it is beyond the scope of this manuscript to investigate whether there are biological examples of dynein oscillating between > 2 cortex locations, we have added to the discussion of oscillatory timing to speculate that, due to a lack of sustained and repetitive dynein activity pulling towards a consistent cortex region, oscillations between >2 cortex locations may disrupt centrosome clustering in a manner consistent with that seen with very quick oscillations between 2 locations.

4. One data set in the manuscript was obtained by imaging live cells expressing fluorescently-tagged tubulin. This, again, seems to be not ideal because information about centrosome number, which is important in the context of this study, is not available in these experiments. The authors argue that these live-cell experiments are necessary to understand whether centrosome clustering occurs late in metaphase (but prior to anaphase onset), which may be underestimated by the fixed-cell analysis. However, analyzing centrosome clustering in both late prometaphase/metaphase and anaphase cells in fixed samples immunostained for tubulin and centrin would provide more informative data than the live-cell experiments presented here.

The fixed cell imaging the reviewer requests to assess centrosome clustering (with visualization of both centrioles and microtubules) is represented in Figures 2 and 3A/B. The fixed imaging data in Figure 2 show that the frequency of clustering is reduced within a population of mitotic cells when Afadin or LGN are depleted. The data in Figure 3A indicate that delaying mitotic exit (via addition of MG132) does not lead to an increase the frequency of clustering.

These data can support two distinct models: that in the absence of Afadin/LGN the majority of cells never achieve clustering, or alternatively, that centrosomes cluster transiently but that this clustering is dynamic/unstable and the steady state of clustering remains low. Additionally, fixed cell imaging can not rule out the possibility that late prometaphase cells with full kinetochore microtubule attachments experience anaphase onset following what would otherwise be transient clustering. Progression into anaphase would deplete such cells from the analysis of mitotic cells performed in Figures 2 and 3A/B.

The live cell imaging we performed addressed these limitations by allowing us to both monitor the *dynamics* of spindle pole clustering and to additionally assess whether clustering may immediately precede anaphase onset. To address the limitation of tracking microtubules (and not centrosomes) that is also highlighted by this reviewer, we had additionally quantified the frequency of centrosome clustering and multipolar divisions in fixed cell images of anaphase cells stained for both microtubules and centrioles. This data was represented in Figure 3F & G.

5. Can the authors show IF images that confirm/show how dynein localization is disrupted after Afadin and LGN KD? Is dynein/NuMa distributed around the entire cortex or entirely absent? Are dynein oscillations affected? All this would be useful information for the model.

The role of LGN/Afadin/NuMA in anchoring dynein to the cortex is well described. Indeed, we chose Afadin and LGN as targets for depletion due to their already characterized role in linking dynein to the cell cortex. In the absence of LGN or Afadin, dynein localization to the cell cortex of mitotic cells is reduced. (Du and Macara, 2004; Kiyomitsu and Cheeseman, 2012; Kotak et al., 2012, 2013; di Pietro et

al., 2016; Okumura et al., 2018; Carminati et al., 2016). Given the loss of dynein localization to the cortex, oscillations would necessarily cease as well.

6. Some of the results in Fig. 3 are difficult to reconcile. Specifically, after 1 hr in MG132 only ~80% of RPE1 indPLK4 have bipolar (clustered) spindles, but in the live cell images experiments the average mitotic duration is 45 min (shorter than the 1 hr MG132 treatement) and ~95% of the cells achieve bipolarity. Also, these numbers seem high for RPE1 cells and inconsistent with findings from other studies (e.g., Chen at al., Mol Biol Cell, 2016 and Baudoin et al., eLife, 2020). Is the level of clustering being overestimated here or is this specific to PLK4-induced centrosome amplification compared to DCB or endoreduplication?

Figure 3A quantifies centrosome clustering in all cells that were in or have entered mitosis since the addition of MG132 and only considers centrosomes clustered if they satisfy the <5um proximity criteria, as described above. The live cell imaging experiments (and corresponding fixed cell analysis of anaphase cells) quantify the fate of cells as they progress through mitosis. This is an important distinction as there is not a 1:1 ratio of spindle number with daughter cells formed. As such, the discrepancy noted by this reviewer can be accounted for by a small fraction of cells entering a bipolar anaphase without meeting our stringent criteria for centrosome clustering-for example, if the cleavage furrow divides the cell such that two distinct but adjacent spindle poles are incorporated into a single daughter cell. This pseudo-bipolar division is common and has been described previously, including in the Baudoin et al 2020 paper where they quantify only 25% of cells with bipolar spindles yet observe that >50% of cells (including some with 3 or 4 spindle poles) complete a division that generates two daughter cells.

Similarly, the frequency of bipolar divisions we describe is comparable to that described in other human cell culture models. For example, in the Chen et al, 2016 paper noted by the reviewer, a frequency of ~87% bipolar divisions in polyploid HeLa cells (that presumably have extra centrosomes) is described in Figure 4D. This frequency is quite similar to our quantification of 95% of RPE indPLK4 cells completing a bipolar division.

7. The model predicts that dynein/NuMA oscillations could be important for clustering centrosomes. GFP-tagged NuMA and live cell imaging could help to test this. Even the current live-cell experiments could be used. The model would suggest that centrosomes closer to the Dynein/NuMA pole would cluster first; then, upon oscillation the other centrosomes may start to cluster. Is there any evidence for this (i.e., spatial/temporal differences in clustering of centrosomes) in the time-lapse videos?

We agree with this reviewer that it would be exciting to experimentally test the model that oscillations in NuMA/dynein cortical localization drive centrosome clustering. However, in order to experimentally distinguish between a correlative and causal role for these oscillations (since dynein activity both moves centrosomes and is in turn inhibited by the centrosomes it moves) this approach would require a mechanism to control oscillations, such as an optogenetic approach to tether NuMA/dynein at specific regions of the cortex that is discussed above, that are beyond the scope of this work. We have expanded the discussion to better highlight the exciting implications that the oscillatory behavior of cortical dynein may have in centrosome clustering.

Minor issues

1. There were many issues with figure/panel numbering and descriptions in figure legends. For instance, some cited figure panels did not exist, figures were note referenced in order, some panels were not described in the figure legends, etc. There were also some labeling issues in the figure themselves (e.g., MDA-MB-231 labeled as MDA-MB-123 in Fig. 2F).

We have reviewed and revised all figure legends, ensuring their proper labeling

2. Line 184, the text "with significant decreases in the pairwise distance between centrosomes to which those microtubules are anchored for the case of dynein in an angular region of pi/2" is not clear.

We have rephrased this statement

3. Line 281-283. The statement "the formation of a bipolar spindle with a given region of dynein enrichment is not sensitive to centrosome number" is not sufficiently supported by the data provided. Simulation data should be provided to show what happens when the number of centrosomes is higher than four. It would also be important to show whether the "optimal" dynein enriched region changes when centrosome number increases (like in figure S4E)

We have revised the text to clarify that our data indicate that formation of a bipolar spindle with a given region of dynein enrichment is not sensitive to centrosome numbers between 2 and 4.

4. Model parameters: how were the values for P_E and P_H chosen? Specifically, why is P_H set to 0.7 and not to 0.5 like P_E? Is there any experimental evidence for such a difference between Eg5 and HSET?

We have added additional detail to the Modeling portion of the methods section to highlight that parameters for HSET and Eg5, originally defined in a prior publication, were additionally validated to ensure clustering frequencines in the base computational model that are comparable to that seen in our experimental system

5. Figure 4A. It would be helpful to label/mark the 0~pi/2 dynein enriched region.

We have added in the pink arc from 0 to pi/2 for both the top and bottom panel of 4A to further indicate the region of dynein enrichment.

RE: Manuscript #E22-07-0296R
TITLE: "Cortical Dynein Drives Centrosome Clustering in Cells with Centrosome Amplification"

Dear Prof. Manning:

Please address remaining concerns of one of the reviewers.

Sincerely,
Alexander Mogilner
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Manning,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 15 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL):
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Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #2 (Remarks to the Author):

Overall, the authors chose to rebut or disregard most of the suggestions made by the reviewers. I will not insist on everything,

but I feel that addressing some of the points I initially raised would improve this manuscript and provide additional insight. Therefore, I listed below a few points that I would like to re-iterate and would like to ask the authors to address.

1. In response to my comment on Plk4 overexpression (major issue #2), the authors provided an answer that simply did not address my question. The authors argued that Plk4 overexpression has been shown to induce centriole overduplication. However, my question was not about whether or not the method they used results in centrosome amplification. So, I will try to explain my point again. Plk4 overexpression is known to result in the production of extra centrosomes, but the number of centrosomes after 48 hrs of doxycycline is typically five or greater in the vast majority of the cell population. So, I was asking to (i) quantify this more accurately (i.e., how many centrosomes per cell); (ii) run simulations with centrosome numbers that match the numbers recorded in the experiments.

2. In response to my major comment #5, the authors argue that the methods they used were shown by others to disrupt dynein localization. In fact, I did not argue otherwise. I was just asking the authors to show that this is, indeed, the case in their hands. I believe it is important to show that the phenotype is consistent with what the authors expect based on previously published work.

3. In my point #7, I asked a very simple question, but the authors' answer went beyond the point. I will try to re-state my question: in the live-cell experiments (Figure 3C), is there evidence that, when dynein is present, centrosome clustering occurs at one end of the cells and is followed, with some delay, by clustering at the other end? This observation would not test the model prediction, but would support it and would be a good point to make in the manuscript.

We thank the reviewer for their thoughtful critique. We have addressed each comment, as detailed below, and feel that the experimental and textual changes suggested by reviewers have improved the clarity of the manuscript.

Point by point response to reviewers:

Reviewer #2 (Remarks to the Author):
Overall, the authors chose to rebut or disregard most of the suggestions made by the reviewers. I will not insist on everything, but I feel that addressing some of the points I initially raised would improve this manuscript and provide additional insight. Therefore, I listed below a few points that I would like to re-iterate and would like to ask the authors to address.

We apologize that our responses were received as disregarding the reviewer's comments. In the previous revision, we did run approximately 500 new simulations to further characterize model simulations with four centrosomes (30 simulations per condition). We also performed additional analysis to quantify cortical dynein, updated figures to include centriole insets, and clarified the text to better highlight requested experiements that had been missed in the supplemental materials. We appreciate all of the feedback and have worked again to address the points brought up. The marked up files have the latest updates in blue font.

Major issues:

1. In response to my comment on Plk4 overexpression (major issue #2), the authors provided an answer that simply did not address my question. The authors argued that Plk4 overexpression has been shown to induce centriole overduplication. However, my question was not about whether or not the method they used results in centrosome amplification. So, I will try to explain my point again. Plk4 overexpression is known to result in the production of extra centrosomes, but the number of centrosomes after 48 hrs of doxycycline is typically five or greater in the vast majority of the cell population. So, I was asking to (i) quantify this more accurately (i.e., how many centrosomes per cell); (ii) run simulations with centrosome numbers that match the numbers recorded in the experiments.

In Supplemental Figure 1, we have added a new panel in D that quantifies the number of centrioles observed in our indPLK4 cells. There is a large range of centrioles, as expected, from 5 to 20. In Figure 3, panel D now quantifies the number of microtubule nucleating centers at mitotic entry, which is primarily in the range of 3 to 8. This is now referred to in the Results section. We agree with the reviewer's point that centriole number is high, but this is the motivation for performing a parallel additional experimental analysis of +DCB and MDA-MB-231 cells.

The manuscript now shows results for simulations with a range of centrosomes from 2 to 6. Supplemental Figure 3 has been updated to show centrosome traces for the case of enriched dynein in a region of pi/2, uniform dynein, and no dynein, for the case of both 5 and 6 centrosomes (D-I). Figure 4 F now quantifies the percent of simulations that form a bipolar spindle for 3 to 6 centrosomes for 30 minutes of simulated time. We now highlight these additional simulations in the results and discussion sections. We note that simulations with more than 6 centrosomes are extremely time intensive and were not completed since our computational model results are in line with centrosome dynamics

quantified in the indPLK4 cells (where many cells had 3 to 6 microtubule nucleating centers), as well as the +DCB with 4 centrosomes and MDA-MB-231 with 2 or more centrioles.

2. In response to my major comment #5, the authors argue that the methods they used were shown by others to disrupt dynein localization. In fact, I did not argue otherwise. I was just asking the authors to show that this is, indeed, the case in their hands. I believe it is important to show that the phenotype is consistent with what the authors expect based on previously published work.

To illustrate the phenotype, we have added a new panel C in Supplemental Figure 1 that shows representative fixed-cell images stained for NuMA and p150 in control and LGN or Afadin depleted cells.

3. In my point #7, I asked a very simple question, but the authors' answer went beyond the point. I will try to re-state my question: in the live-cell experiments (Figure 3C), is there evidence that, when dynein is present, centrosome clustering occurs at one end of the cells and is followed, with some delay, by clustering at the other end? This observation would not test the model prediction, but would support it and would be a good point to make in the manuscript.

While we do occasionally see examples of staggered clustering in our live cell imaging of GFP-tubulin movies (where a cell progresses from four to three and then to two spindle poles), the temporal resolution (images were captured at 5 min intervals) and lack of a centriole/centrosome marker preclude us from making the spatial and temporal measurements needed to have a clear picture of when, with respect to cortical positioning, centrosomes clusters.

RE: Manuscript #E22-07-0296RR
TITLE: "Cortical Dynein Drives Centrosome Clustering in Cells with Centrosome Amplification"

Dear Prof. Manning:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Alexander Mogilner
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Manning:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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